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PHENOL TOLERANCE AND DEGRADATION PROFILE OF NOVEL EDIBLE MUSHROOM *HYPSIZYGUS ULMARIUS* IN LIGNINOLYTIC AND NON-LIGNINOLYTIC MEDIA

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ABSTRACT

Phenol tolerance and degradation using spent mycelium substrate of *Hypsizygus ulmarius* showed that ligninolytic media with carbon or nitrogen limitation gave highest growth (26.27sq.cm), while limitation of both nutrients reduced it to 8.1sq.cm. 7.68sq.cm was seen in non-ligninolytic media; simultaneous nutrient increase gave 1.16sq.cm. Tolerance to phenol was best in ligninolytic media with carbon or nitrogen limitation. When phenol was introduced, growth decreased. Phenol increase from 200 to 400mg/L caused further decrease in nitrogen-optimum(7.03 to 2.35 sq.cm) and deficient(6.33 to 1.23 sq.cm) media, while nitrogen-deprived (2.48 to 5.52 sq.cm) media showed increase. Tolerance to higher phenol concentrations was accentuated by severe nitrogen limitation. Correlation of 0.99 between ligninolytic and non-ligninolytic media showed growth pattern similar irrespective of nutrient levels. Nitrogen reduction achieved 63% degradation from 200-800mg/L phenol, while carbon limitation gave 55.75%. Negligible degradation in non-ligninolytic condition. At lower phenol concentration, nitrogen limitation beneficial; for higher concentrations, carbon limitation helped.

KEYWORDS: ***Hypsizygus ulmarius*, Ligninolytic media, Non-ligninolytic media, Phenol Tolerance and Degradation Profile, Spent Mycelium Substrate**.

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INTRODUCTION

Phenol is a priority pollutant; listed in EPA's Priority Pollutant List[11]. The origin of phenol in environment is both industrial and natural. Industrial phenol pollution is associated with pulp and coal mines, refineries, wood preservation plants, etc.[29]. Bisphenol A (BPA) is produced at over 6 billion pounds per year, one of the highest produced commercial products. It is used to manufacture polycarbonate plastic products, resins lining metal cans, dental sealants and blends with other types of plastic products[4]. Heat and contact with acids or bases accelerate hydrolysis of ester bonds linking BPA molecules in polycarbonate and resins, resulting in increase in leaching rate[16]. Pentachlorophenol (PCP) is lethal to a wide variety of plants and animals, as an inhibitor of oxidative phosphorylation. Of all PCP produced, 80% is used by wood preserving industry as a pesticide[10]. There is evidence that suggests definite accumulation of PCP through the food chain[27]. PCP is thought to be mutagenic, or at least co-mutagenic, and human exposure poses significant health hazards[31]. Use of fungi for bioremediation of phenolic compounds necessitates an understanding of variables that enhance their ability to detoxify these recalcitrant molecules and the enzymatic pathways used[6]. This requires studying their tolerance and degradation capability to the precursor chemical (viz.) phenol.

Most research has focused only on *Phanerochaete chrysosporium*[12]. However, many other species have similar or enhanced capabilities. *Pleurotus ostreatus* can transform Bisphenol A[33] upto 80% effectiveness during 12 days incubation[24]. Here, laccase (Lac) was considered primarily responsible for oxidation of phenolics and aromatic amines, by reducing molecular oxygen to water[23]. *Lentinula edodes* transforms pentachlorophenol by reductive dehalogenation and o-methylation reactions[25], chlorinated aminophenols and chloro hydroquinone[35]. Tolerance and degradation of phenol occurs in white-rot fungi, a physiological group of basidiomycetes with proven lignin-degrading capacities, due to secretion of phenol oxidase enzymes like lignin peroxidase, manganese peroxidase or laccase[36,37]. Polyphenol oxidase is a monoxygenase which catalyses the o-hydroxylation of phenols and oxidation of o-dihydric phenols to o-quinones using molecular oxygen. Laccases are phenol oxidases which utilize molecular oxygen, oxidizing polyphenols, meta substituted phenols, diamines and other components[17]. The relevance of this study is that mushroom cultivation is a highly efficient method of disposing agricultural residues as well as producing nutritious food[7]. The cultivation of *Pleurotus spp.* is ranked second or third in the world[9].

For every kilogram of mushroom produced, 5 kg of SMS is generated[34]. Spent Mycelium Substrate (SMS) is a valuable by-product of edible mushroom cultivation. It consists of partially degraded paddy or wheat straw, coconut husk or other agricultural wastes. After a few cultivation cycles, it is bio-chemically modified by fungal enzymes into a simpler form, enriched with protein. SMS is considered as a part of solid waste, and the mushroom industry is facing pressure from regulatory agencies to use SMS generated in a more environmental friendly manner (viz.) bioremediation or as biofertilizer[28], than by simply burning it, as is being done. *H.ulmarius* is a novel species of edible mushroom developed for the climate of Bangalore city, by Indian Institute of Horticultural Research (IIHR). It can be easily cultivated on various common natural substrates like paddy straw[13], coconut husk, tea dust, sawdust and sugarcane bagasse[2]. Most fungi are known to degrade cellulosic substrates, especially basidiomycetes, with nearly 1,500 species[21].

As per The Hazardous Waste Management Rules, 1989, only 5 kg phenol is permitted by law for disposal per year. Actual quantities being disposed off are much greater. Also, present treatment methods for phenol are highly chemical intensive that further degrades the

environment. This makes it essential that better and non-chemical methods, such as mycoremediation are developed[5]. The objective of the study was to make a preliminary assessment of phenol tolerance and degradation profile of *H.ulmarius* under variations of carbon and nitrogen in growth media and increasing concentrations of phenol to understand in future, its potential for bioremediation of phenolic wastes in contaminated sites with marginal fertility levels.

MATERIALS AND METHODS

TOLERANCE STUDIES:

The spawn of *H.ulmarius* was sourced from IIHR, Bangalore and cultured on Mandel and Weber's modified agar medium[22] for studying its tolerance to phenol. Carbon source was glucose. Nitrogen content was varied by changing quantities of urea, ammonium sulphate and peptone. Phenol was 99% pure and sourced from Fischer Scientific Co. Concentrations tested were 200, 400 and 800mg/L of culture media. Media variations were nitrogen-deficient (N/2), nitrogen-deprived (N/10), carbon-deficient(C/2), carbon-deprived(C/10), nitrogen carbon-deprived(N/10,C/10), nitrogen-excess(Nx10), carbon-excess(Cx10) and nitrogen carbon-excess(Nx10,Cx10). Tolerance was estimated by measuring rate of increase of mycelial radius every alternate day, for a total of 22 days[38].

DEGRADATION STUDIES:

The spawn of *H.ulmarius*, sourced from IIHR, Bangalore, was cultivated on paddy straw by solid-state fermentation (SSF) technique[8]. SSF is considered as the most appropriate method for filamentous fungi cultivation and ligno-cellulolytic enzyme production because they grow under conditions close to their natural habitats, due to which they are more capable of producing certain enzymes and metabolites, which usually will not be produced or produced only at low yield in submerged cultures.

CULTIVATION OF HYPSIZYGUS ULMARIUS [8]:

SUBSTRATE PREPARATION AND STERILIZATION:

The substrate was chopped paddy straw, generally used for *Pleurotus* cultivation in Asia[14]. It contains 41% cellulose, 13% lignin, 0.8% total N, 0.25% P_2O_5, 0.3% K_2O, 6% SiO_2, pH 6.9, C/N = 58 [15]. Straw was cut to length of 2-5 cm, soaked overnight in water and steam sterilized in autoclave, for 1 hour at 65^oC.

SPAWNING AND SPAWN RATE:

The amount of spawn used was 5% of total weight of substrate (50 gm spawn per kg substrate).

FILLING PLASTIC BAGS WITH SUBSTRATE:

Pasteurized substrate was spawned and filled into clear perforated polyethylene bags; incubated at 23-25^oC for 12-14 days. Mushrooms started to form around the edges of bag perforations and harvested 3 to 4 weeks after spawning.

PRODUCTION CONDITIONS:

After spawning, bags were moved to a room where air temperature was maintained at 18–20^oC. Relative humidity maintained at 95-98%. The first 12-21 days of spawn run were completed without artificial lighting, after which 4 hours of light was provided daily. At the time of pinning (mushroom formation), sufficient air was introduced to lower CO_2 levels.

USE OF SPENT MYCELIUM SUBSTRATE FOR MYCOREMEDIATION:

After 2-3 cycles of mushroom harvesting, spent mycelium substrate was dried completely, separated into fibres and used for liquid phenol degradation studies[28]. Degradation estimated by phenol assay using Folin's reagent method, for 6 days after inoculation.

STATISTICAL ANALYSIS:

All variations were tested in triplicate, with average values computed. Correlation was

determined between variations of nitrogen and carbon in media and increasing phenol concentrations. The Statistical validation was done using MS Excel software.

RESULTS AND DISCUSSION

Ligninolytic media with carbon or nitrogen limitation (N/2, N/10, C/2, C/10) showed highest average mycelial growth of 26.27sq.cm, while simultaneous limitation reduced it to 8.1sq.cm (Figure 1). Even lesser growth of 7.68sq.cm seen in non-ligninolytic media (Nx10 or Cx10); simultaneous increase of nutrients resulted in growth of 1.16sq.cm (Figure 2).Thus, tolerance to phenol was greatest in ligninolytic media with carbon or nitrogen limitation. Primary growth ceases when nitrogen is depleted, and ligninolytic activity subsequently appears after a lag period[19].

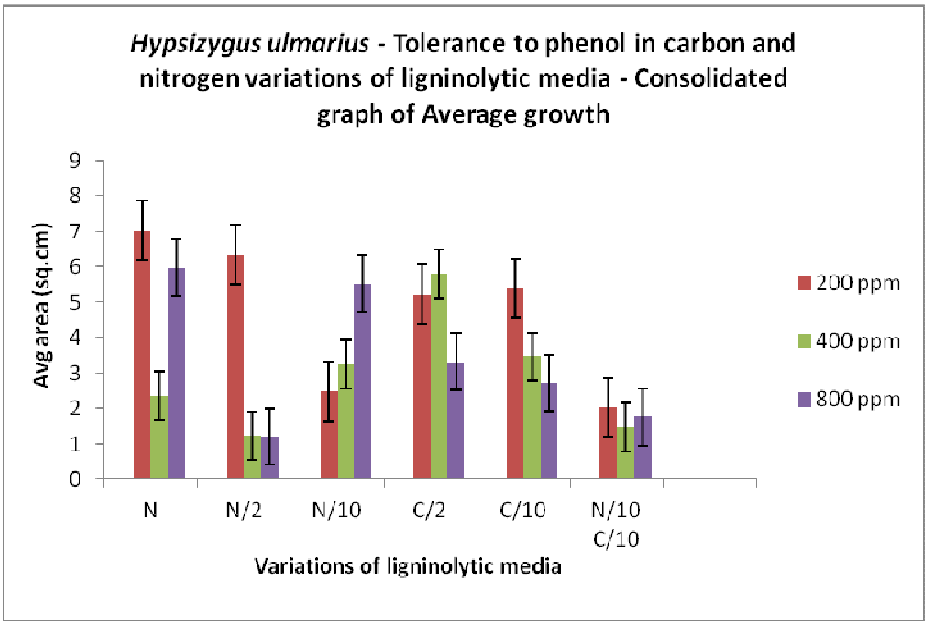

Figure 1
Tolerance profile of H.ulmarius to phenol in carbon and nitrogen variations of ligninolytic media

When phenol introduced at 200mg/L, growth decreased except in non-ligninolytic media (Nx10,Cx10 and Cx10). Glucose, a simple sugar, was utilized without breakdown, thus growth of *H.ulmarius* using glucose was well supported. Glucose and fructose were the most readily utilized carbohydrate sources for growth of *Pleurotus tuber-regium and P. squarrosulus* respectively.[1,18] Phenol increase from 200 to 400mg/L caused further decrease in nitrogen optimum (7.03 to 2.35 sq.cm) and deficient(6.33 to 1.23 sq.cm) media, while nitrogen deprived(2.48 to 5.52 sq.cm) media showed increase. Thus, tolerance to higher phenol concentrations increased under severe nitrogen limitation (Figure 1,2). When phenol concentrations were low to moderate in media (200 and 400mg/L), nitrogen sufficient condition offered better tolerance and growth to *P. florida*, but for high phenol concentration (800mg/L), nitrogen depleted, deprived and excess conditions provided the same benefit[32].

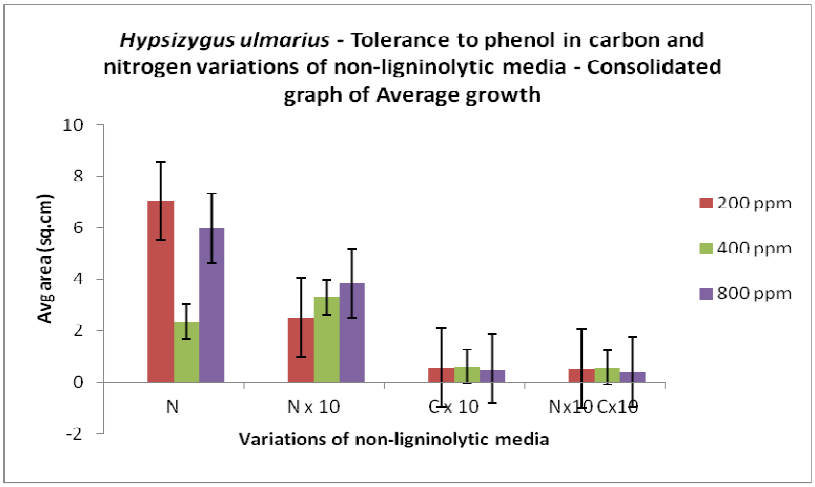

Figure 2
Tolerance profile of H.ulmarius to phenol in carbon and nitrogen variations of non-ligninolytic media

Correlation of 0.946 between growth in ligninolytic and non-ligninolytic media showed growth pattern similar irrespective of nitrogen or carbon levels. There was no significant correlation between growth patterns at varying phenol concentrations (Table 1).

Table 1
Correlation of growth of H.ulmarius between different phenol concentrations and nitrogen variations of media

***H.ulmarius* - Correlation between growth patterns in varying phenol concentrations**		
200 and 400 ppm	**400 and 800 ppm**	**200 and 800 ppm**
0.331	0.581	0.651

***H.ulmarius* - Correlation between growth patterns in ligninolytic and non-ligninolytic media**	
B/w N/2 and N x 10	0.964202114
B/w N/2 and Nx10 Cx10	0.967496523
B/w N/10 and Nx10 Cx10	0.915329737
B/w Nx10 and N/10 C/10	0.988707107
B/w N/10 C/10 and Nx10 Cx10	0.964066372
B/w C/2 and C x 10	0.926635474
B/w C/10 and C x 10	0.898399382

Reduction of nitrogen (N/2, N/10) achieved 30% degradation at 200mg/L; 23% at 400mg/L and 10% at 800mg/L. Limitation of carbon (C/2, C/10) resulted in 21.25%, 18% and 16.5% degradation from 200 to 800mg/L. Non-ligninolytic media (Nx10 or Cx10) achieved 10% degradation at 200mg/L and negligible at higher concentrations (Figure 3).

Thus, higher phenol concentration was degraded better under carbon limitation while nitrogen limitation offered the same advantage for lower concentration. Degradation favourable under ligninolytic condition; however combined limitation reduced degradative capability (Figure 3). Low nitrogen concentration in medium enhanced enzymatic capability of most white rot basidiomycetes[20]. Lignin degradation is a secondary metabolic event and de novo synthesis of a typical secondary metabolite veratryl alcohol, begins simultaneously with the onset of ligninolytic activity in nitrogen-starved cultures of *Phanerochaete chrysosporium*[26]. Veratryl alcohol is the prime intermediate during laccase degradation of phenolic substrates.

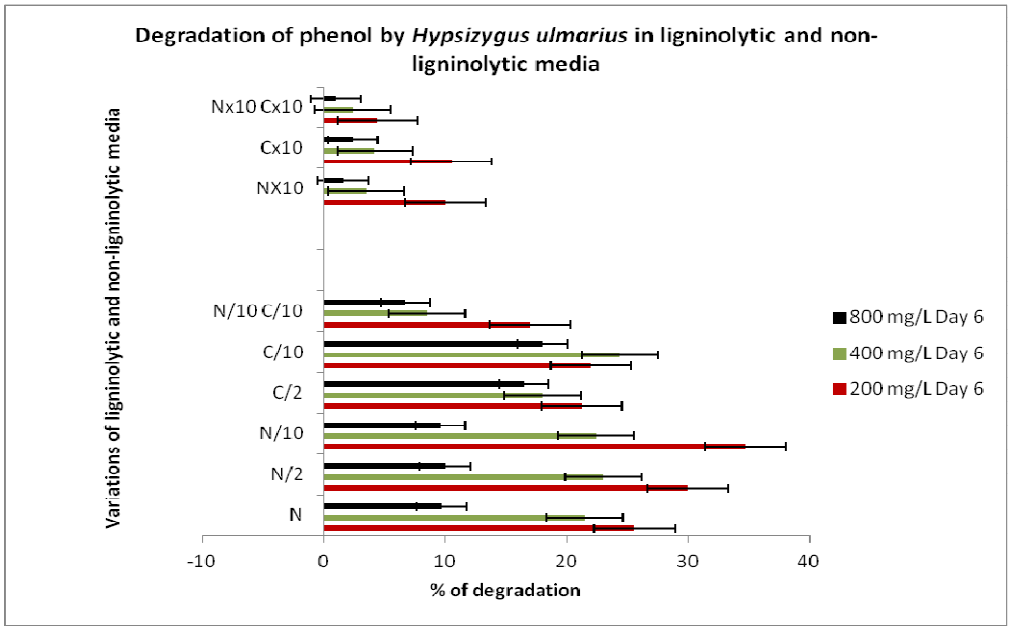

Figure 3
Degradation profile of H.ulmarius in carbon and nitrogen variations of ligninolytic and non-ligninolytic media

CONCLUSIONS

Reduction of nitrogen or carbon content gave increased mycelial growth, thus cultivation of *Hypsizygus ulmarius* in marginal soils, especially contaminated sites (where plant nutrients like carbon and nitrogen are limiting factors) is practically feasible. Ligninolytic media also enhanced tolerance to higher concentrations of phenol, thus *H.ulmarius* growing on nitrogen-deficient marginal soils has a greater potential for bioremediation, as tolerance to higher concentrations of a contaminant is one of the prime requisites for a micro-organism considered as suitable for bioremediation. However, combined limitation of carbon and nitrogen reduced growth drastically. Higher phenol concentrations were degraded better under carbon limitation. In most fermentation processes, increasing glucose concentration has a suppressive effect on production of bioactive compounds and growth[30]. However, lower concentrations of phenol degraded better under nitrogen limitation. Lignin and phenol degradation is a secondary metabolic event triggered by limitation of carbon, nitrogen or sulphur[3]. Combined limitation reduced degradation.

REFERENCES

1. Alofe FV., The general characteristics and cultivation of some Nigerian mushrooms, Ph.D thesis, Obafemi Awolowo University, Ile Ife Nigeria, (1995).
2. Beig G., Jandaik C., Artificial cultivation of *Pleurotus cystidiosus* in India, Mushroom Science, 12(2): 67-71, (1989).
3. Broda P., Birch PRJ., Brooks PR., Sims PFG., Lignocellulose degradation by *Phanerochaete chrysosporium*: gene families and gene expression for a complex process, Mol. Microbiol., 19, 923–932, (1996).
4. Burridge E., Bisphenol A: product profile, Eur Chem News., 17, 14-20, (2003).
5. C.A. Wentz, Hazardous Waste Management, II Ed., McGraw Hill Int. Edition, Chemical Engineering Series, McGraw Hill Inc., Singapore: 80-86, 296-351, (1995).
6. C.E. Cerniglia, J.B. Sutherland, S.A. Crow, Fungal metabolism of aromatic hydrocarbons. In: G. Winkelmann, (Ed.), Microbial Degradation of Natural Products, VCH Press, Weinheim, 1992, p. 193–217.
7. Chang ST., Buswell JA., Mushroom Nutriceuticals, World J. Microbiol. Biotechnol., 12, 473–476, (1996).
8. Chang ST., Lau OW., Cho KY., The cultivation and nutritive value of *Pleurotus sajor –caju*. European J. Appl. Microbiol. Biotechnol., 12: 58–62, (1981).
9. D. Moore, S.W. Chiu, In: S.B. Pointing, D. Hyde. (Eds.) Exploitation of filamentous fungi, Fungal Diversity Press, Hong Kong, 2001, p. 223-252.
10. D.D. Kaufman, Degradation of pentachlorophenol in soil, and by soil microorganisms, In: K. R. Rao (ed.), Pentachlorophenol: chemistry, pharmacology, and environmental toxicology. Plenum Publishing Corp., New York: 1977, pp. 27-39.
11. EPA., Phenol ambient water quality criteria. Office of the planning and standards. Environ. Protect. Agency, Washington, DC, 296-786, (1979).
12. George EJ., Neufeld RD., Degradation of fluorene in soil by the fungus *Phanerochaete chrysosporium.* Biotechnol. Bioeng., 33: 1306-1310, (1990).
13. Hanscha C., McKarnsb SC., Smith CJ., Doolittle DJ., Chemico-Biological Interactions, 127(1): 61–72, (2000).
14. Hashimoto K., Takahashi Z., Studies on the growth of *Pleurotus spp.,* Mush. Sc., 9(2): 585-593, (1976).
15. Heltay I., Zavodi I., Rice straw compost, Mush. Sc., 4, 393-399, (1960).
16. Howdeshell KL., Peterman PH., Judy BM., Taylor JA., Orazio CE., Ruhlen RL., Bisphenol A is released from used polycarbonate animal cages into water at room temperature, Environ Health Perspect, 111, 1180-1187, (2003).
17. Kadhim H., Graham C., Baratt P., Evane CS., Rastall RA., Removal of phenolic compounds by *Coriolus versicolor* grown on wheat bran, Enzyme Microbial. Technol., 24: 303-307, (1999).
18. Kadiri M., Physiological studies on some Nigerian mushrooms. Ph.D thesis, University of Ibadan, Nigeria, (1990).
19. Keyser P., Kirk TK., Zeikus JG., Ligninolytic enzyme system of *Phanerochaete chrysosporium* synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol., 135: 790-797, (1978).
20. Kirk TK., Connors WJ., Bleam RD., Hackett WF., Zeikus JG., Proceedings of National Academy of Science, U.S.A., 72, 2515-2519, (1975).
21. M.J. Carlile, S.C. Watkinson, The fungi, Academic Press, New York, N.Y: 269–275, (1997).
22. Mandels M., Weber J., Adv. Chem. Ser., 95, 391-414, (1969).
23. Martirani L., Giardina P., Marzullo L., Sannia G., Reduction of phenol content and toxicity in olive oil mill wastewaters with the ligninolytic fungus *Pleurotus ostreatus*, Water Res., 30: 1914–1918, (1996).

24. Mirano T., Monda Y., Watanab T., Kuwhara M., Degradation of Bisphenol A by the lignin-degrading enzyme, manganase peroxidase produced by the white-rot basidiomycete, *Pleurotus ostreatus*, Biosci. Biotechnol. Biochem., 64, 1958-1962, (2000).
25. Okeke B., Paterson A., Smith J., Watson-Craik L., Comparative biotransformation of pentachlorophenol in soils by solid substrate cultures of *Lentinula edodes.* Appl. Microbiol. Biotechnol, 48: 563-569, (1997).
26. P. Fenn, T.K. Kirk, Formation and action of the ligninolytic system in basidiomycetes, In: J. Hedger and J. Frankland (ed.), Decomposition by basidiomycetes, Cambridge University Press, Cambridge, 1980, pp. 152-169.
27. P. Lu, R. L. Metcalf, L. K. Cole, The environmental fate of '4C-pentachlorophenol in laboratory model ecosystems, In: K. R. Rao (ed.), Pentachlorophenol: chemistry, pharmacology, and environmental toxicology. Plenum Publishing Corp., New York: 1977, pp 53-63.
28. Padmavathi Tallapragada., Ranjini Rajiv., Vedha Anjanappa., Sehaprabha Sardesai., Samrudhi Selvaraj., Shahid Khan., Comparing the potential of spent mycelium substrate of *Pleurotus florida* with biofertilizers to enhance growth of *Capsicum annuum.* Asian J. of Plant Science and Research, 1(4): 76-86, (2011).
29. Paula M., Van Schei., Young LY., Isolation and Characterization of phenol degrading denitrifying bacteria. Appl. Environ. Microbiol., 64(7): 2432-2438, (1998).
30. R. Hutter, Design of culture media capable of provoking wide gene expression. In: J.D. Buluock, L.J. Nisbet, D.J. Winstanley, (Eds.) Bioactive microbial products, Search and Discovery Academic Press, London, 1982, pp. 37-50.
31. R.C. Dougherty, Human exposure to pentachlorophenol, In: K. R. Rao (ed.), Pentachlorophenol: chemistry, pharmacology and environmental toxicology. Plenum Publishing Corp., New York: 1977, pp. 351-361.
32. Ranjini R., Padmavathi T., Phenol tolerance of *Pleurotus florida* under varying conditions of nitrogen sufficiency, Eur. J. of Experimental Biology, 2(1): 75-82, (2012).
33. Saito T., Kato K., Yokogawa Y., Nishida M., Yamashita N., Detoxification of bisphenol A and nonylphenol by purified extracellular laccase from a fungus isolated from soil, Journal of Bioscience and Bioengineering, 98(1): 64-66, (2004).
34. Semple KT., Reid BJ., Fermor TR., Review of composting strategies to treat organic pollutants in contaminated soils, Environmental Pollution, 112, 269-283, (2001).
35. Stazi S., Annibale A., Sermanni G., Kinetic behaviour and degradative capability of *Lentinula edodes* laccase isoenzymes with differently substituted compounds, (2002).
36. Tanaka H., Itakura S., Enoki A., Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *P.chrysosporium*, Holzforschung, 53, 21–28, (1999a).
37. Tanaka H., Itakura S., Enoki A., Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *T.versicolor*, J. Biotechnol., 75, 57–70, (1999b).
38. Toure, P.S. Chahal, M Ishaque, D.S. Chahal, In: Global Environmental Biotechnology, 1997, pp. 649-664.
39. www.iihr.res.in/section-of-seedscience-and-technology/03Aug 2012